

Antigenic Subunits of Hantaan Virus Expressed by Baculovirus and Vaccinia Virus Recombinants

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Baculovirus and vaccinia virus vectors were used to express the small (S) and medium (M) genome segments of Hantaan virus. Expression of the complete S or M segments yielded proteins electrophoretically indistinguishable from Hantaan virus nucleocapsid protein or envelope glycoproteins (G1 and G2), and expression of portions of the M segment, encoding either G1 or G2 alone, similarly yielded proteins which closely resembled authentic Hantaan virus proteins. The expressed envelope proteins retained all antigenic sites defined by a panel of monoclonal antibodies to Hantaan virus G1 and G2 and elicited antibodies in animals which reacted with authentic viral proteins. A Hantaan virus infectivity challenge model in hamsters was used to assay induction of protective immunity by the recombinant-expressed proteins. Recombinants expressing both G1 and G2 induced higher titer antibody responses than those expressing only G1 or G2 and protected most animals from infection with Hantaan virus. Baculovirus recombinants expressing only nucleocapsid protein also appeared to protect some animals from challenge. Passively transferred neutralizing monoclonal antibodies similarly prevented infection, suggesting that an antibody response alone is sufficient for immunity to Hantaan virus.

Hantaan virus, the etiologic agent of Korean hemorrhagic fever, is the prototype of the *Hantavirus* genus of the *Bunyaviridae* family. Korean hemorrhagic fever and clinically similar diseases, collectively termed hemorrhagic fever with renal syndrome (HFRS), pose a significant health threat in much of Asia and parts of Europe and Scandinavia. In the People's Republic of China, an estimated 50,000 to 100,000 cases occur annually, with mortality rates reported to be 5 to 20% in various provinces (6, 23). A vaccine for HFRS is not yet available, although inactivated Hantaan virus-infected mouse brain or rat brain preparations have been tested in humans in North Korea and South Korea (7, 26; D. J. Suh, J. W. Song, and H. W. Lee, *Virus Information Exchg. Newsl.* 6:131, 1989). Inactivated tissue-culture-grown Hantaan virus vaccines are under development and testing in Japan and China (25; U.-X. Yu and Z.-Y. Zhe, *Virus Information Exchg. Newsl.* 6:131, 1989). Since Hantaan virus was isolated from the lungs of the Korean striped field mouse, *Apodemus agrarius corea*, by Lee et al. in 1978 (9), vaccine development efforts have been hampered by difficulties in virus propagation in a number of cell lines, the slow and low-titer replication of virus in cultured cells, the necessity for a containment laboratory for routine studies with infectious virus, and the absence of an animal model for testing the efficacy of potential immunogenic preparations. For vaccine development studies, we attempted to circumvent some of the problems associated with the use of authentic Hantaan virus by using baculovirus and vaccinia virus recombinants expressing the structural proteins of Hantaan virus.

Like other viruses in the *Bunyaviridae* family, Hantaan virus has a three-segmented, single-stranded RNA genome whose segments are designated as large (L), medium (M), and small (S) (15, 17). The S genome segment encodes the viral nucleocapsid protein (N) in the virus-complementary sense RNA, and the M genome segment encodes the enve-

lope glycoproteins G1 and G2 in a single, continuous open reading frame, also in the virus-complementary sense RNA. Unlike viruses in other genera of the *Bunyaviridae*, neither the S nor the M segments of Hantaan virus appears to encode additional nonstructural polypeptides (19, 21). The envelope glycoproteins are presumed to be the major elements involved in induction of immunity to Hantaan virus because monoclonal antibodies (MAb) to G1 and G2, but not to N, have been found to neutralize viral infectivity in vitro (1, 5). The importance of the humoral response for protection against infection was demonstrated with rats by passive transfer of immune sera and subsequent challenge with the hantavirus Sapporo rat (SR-11) virus (27). A cell-mediated immune response to Hantaan virus has also been implicated in protection; however, the actual importance and protein specificity of the response have not been resolved (2, 3).

To investigate the potential efficacy of a recombinant vaccine for HFRS, we analyzed the antigenicity and immunogenicity of expressed Hantaan proteins and developed an animal model to estimate protective immunity. To evaluate the components of a vaccine that might be important for protection, we tested recombinant viruses that expressed the complete M and S segments of Hantaan virus or portions of the M segment containing coding information only for G1 or G2. Our results suggest that immunization with Hantaan virus proteins produced by either recombinant baculoviruses or vaccinia viruses can elicit antibodies and protect animals from infection with Hantaan virus and that Hantaan virus subunit vaccines may provide efficacious alternatives to killed virus vaccines.

MATERIALS AND METHODS

Viruses, cells, and media. *Autographa californica* nuclear polyhedrosis virus (AcNPV) was kindly supplied by Max Summers. *Spodoptera frugiperda* (SF-9) (ATCC CRL 1711) cells were purchased from the American Type Culture Collection, Rockville, Md., and were maintained as suspension cultures in TNM-FH medium (24) supplemented with

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10% fetal bovine serum, 50 μ g of gentamicin per ml, and 50 μ g of Fungizone (GIBCO Laboratories, Grand Island, N.Y.) per ml. The Connaught human vaccine strain of vaccinia virus, which originated from the New York City Board of Health strain, was plaque purified three times on monolayers of MRC-5 human diploid lung cells (ATCC CCL 171). The mouse neurotropic WR strain of vaccinia virus (ATCC VR-119) was originally obtained from the American Type Culture Collection but had undergone limited plaque purification (4) and was provided by Shiu-Lok Hu (Oncogen, Seattle, Wash.). Hantaan virus strain 76-118 was propagated and assayed in Vero E6 cells (Vero C1008; ATCC CRL 1586), which were maintained in Eagle minimal essential medium with Earles salts (EMEM) containing 10% fetal bovine serum and 50 μ g of gentamicin per ml.

Construction of transfer vector plasmids. *Bgl*II restriction sites were positioned near the 3' and 5' termini of cDNA representing the Hantaan virus M genome segment by site-directed mutagenesis (14). Digestion with *Bgl*II generated a restriction fragment of approximately 3.5 kilobases which contained the entire coding region for the Hantaan virus G1 and G2 envelope glycoproteins. The cDNA was cloned into the *Bam*HI site of the baculovirus transfer vectors pAcYM1 (13) or pVL941 (10). For subcloning into the *Sma*I site of the vaccinia virus transfer vector pSC11 (11), the DNA was treated with the large (Klenow) fragment of DNA polymerase I to produce blunt ends. Restriction fragments containing only G1-coding information (*Bgl*II-*Dra*I) or only G2-coding information (*Hpa*II-*Bgl*II) were similarly treated to produce blunt ends and were cloned into the *Bam*HI site of pAcYM1 or the *Sma*I site of pSC11 after treatment of the linearized plasmids with the Klenow fragment. cDNA representing the S genome segment of Hantaan virus, which had been previously cloned into pGem1 (Promega Biotec, Madison, Wis.) (19), was excised by *Acc*I digestion, which cleaves six nucleotides from the 5' end of the message sense Hantaan S cDNA and within the multiple cloning region of pGem1 beyond the 3' terminus of the Hantaan virus cDNA insert. The restricted DNA was treated with Klenow fragment and cloned into the *Sma*I site of pSC11. Construction of the transfer vector pACYM1-Hantaan S was previously described (22).

Recombination with AcNPV and vaccinia virus. Baculovirus recombinations were performed by cotransfecting 3×10^6 SF-9 cells with 1 μ g of purified AcNPV DNA and 20 μ g of transfer vector plasmid DNA, as previously described (24). Cultures were incubated at 27°C until greater than 50% of cells displayed visible polyhedra (after 4 to 6 days), after which cell culture fluids were removed and clarified by low-speed centrifugation, and supernatants were stored at 4°C. Supernatants were diluted to 10^{-5} and 10^{-6} in TNM-FH; and 25, 50, or 100 μ l of each dilution was added to the wells of individual microtiter plates containing SF-9 cells. The plates were incubated for 4 to 6 days, after which cell culture fluids were transferred to new microtiter plates and infected cells were lysed with NaOH and neutralized as previously described (24). The cell lysates were transferred to GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, Mass.) by using a Schleicher & Schuell Hybridot apparatus. Recombinants were identified by hybridization with a cDNA probe representing the M segment of Hantaan virus, which was radiolabeled by random primer synthesis (Pharmacia Fine Chemicals, Piscataway, N.J.). Cell culture media from hybridization-positive wells were diluted to 10^{-6} and 10^{-7} in TNM-FH and used to infect SF-9 cells in microtiter plates as before. Recombinants were again

selected by hybridization. Tissue culture flasks (25 cm²) containing 3×10^6 SF-9 cells were infected with 10 μ l of medium from hybridization-positive wells and were incubated at 27°C for 4 to 7 days. Cells were examined for the presence of polyhedra. Media from cultures that appeared to be free of polyhedra were used as virus stocks for expression studies. The recombination of transfer vectors containing Hantaan virus S segment cDNA and AcNPV has been described previously (22).

For vaccinia virus recombinations, confluent monolayers of Vero E6 cells in 25-cm² flasks were infected at a multiplicity of infection of 0.03 PFU per cell with either the Connaught or WR strain of vaccinia virus diluted in EMEM. After incubation at 37°C for 4 h, the medium was removed and 2 ml of fresh EMEM was added. Prior to transfection of the infected cells, 20 μ g of the plasmid transfer vector was diluted to 0.5 ml in a buffer containing 150 mM NaCl, 0.7 mM Na₂HPO₄, 5 mM KCl, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 6 mM dextrose, adjusted to pH 7.05. Calcium chloride (25 μ l of 2.5 M) was added slowly, and the solution was incubated at room temperature for 0.5 to 1 h. Transfection mixture was added dropwise to the infected cell culture supernatants, and cells were incubated at 37°C for 3 h, after which the medium was removed and fresh EMEM was added. The cells were incubated at 37°C until cytopathic effects were extensive and most of the cells had detached from the flask (4 to 5 days). Cells were then pelleted from the cell culture supernatant by centrifugation (8,000 \times g) and suspended in 10 mM Tris hydrochloride, pH 8.8. After two cycles of freezing at -70°C and thawing at 37°C, cells were sonicated on ice four times for 30 s each at maximum output in a cup sonicator. Plaque assays were performed by incubating dilutions of the disrupted cells on confluent monolayers of Vero E6 cells for 1 h, overlaying the cells with 1% SeaKem agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) in basal medium Eagle (BME; GIBCO) containing 10% fetal bovine serum, 50 μ g of gentamicin per ml, and 0.5 μ g of Fungizone per ml, and incubating them at 37°C. After 3 days, a second agarose overlay, as above, was added with the addition of 300 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Sigma Chemical Co., St. Louis, Mo.) per ml. Recombinants were recovered from blue plaques with a Pasteur pipette and were diluted in EMEM, sonicated, and plaque assayed as above until only blue plaques were observed. To ensure purity of the recombinant viruses, a final plaque was selected from an assay performed with sonicated virus that had been passed through a 0.45- μ m-pore-size cellulose nitrate filter to remove virus aggregates. Virus stocks were prepared from recombinant-infected Vero E6 cells by removing cell culture supernatants when the cytopathic effect was well advanced, scraping cells into sterile phosphate-buffered saline (PBS), pelleting cells at 8,000 \times g for 30 min, and suspending the pellets in sterile 10 mM Tris hydrochloride, pH 8.8. Cells were disrupted in a stainless steel Dounce homogenizer, and cell debris was removed by low-speed centrifugation (200 \times g).

Radiolabeling, immune precipitation, and PAGE. Confluent monolayers of Vero E6 cells in 25-cm² flasks were infected with vaccinia virus recombinants at a multiplicity of infection of approximately 10. At 23 h postinfection, the cell culture medium was removed and replaced with cysteine-free EMEM containing 2% fetal bovine serum. Cells were radiolabeled from 24 to 28 h postinfection with 100 μ Ci of L-[³⁵S]cysteine (Dupont, NEN; >600 Ci/mM) per ml. Cells were lysed on ice with buffer containing 4% Zwittergent

(Calbiochem-Behring, La Jolla, Calif.), and proteins were immune precipitated with polyclonal antibodies or with MAb as previously described (21). SF-9 cells were infected with recombinant baculoviruses and radiolabeled, and proteins were immune precipitated from cell lysates in the same manner, except that the labeling medium was cysteine-free Grace's insect medium (GIBCO) supplemented with 2% fetal bovine serum. To perform polyacrylamide gel electrophoresis (PAGE), immune-precipitated, expressed proteins were subjected to discontinuous electrophoresis on 12.5% acrylamide-*N,N'*-diallyltartardiamide gels as previously described (17).

Passive protection studies with MAb to Hantaan virus. Mouse ascitic fluids (0.2 ml) containing Hantaan-specific MAb were passively administered intraperitoneally (i.p.) to groups of four female 8- to 10-week-old outbred Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Normal mouse ascitic fluids were obtained from outbred ICR female mice with ascites induced by injection of sarcoma 180 cells (ATCC CCL8) i.p. Hamsters were challenged 24 h later by the intramuscular (i.m.) injection of 10^3 PFU of Hantaan virus. Animals were euthanized at 28 days postchallenge, and frozen sections of lungs and kidneys were examined by the indirect fluorescent antibody test (IFAT) for the presence of Hantaan virus-specific antigen. Hamster sera were examined for polyclonal Hantaan virus antibody responses by both plaque reduction-neutralization tests (PRNT) and IFAT with infected Vero E6 cell spot slides (1, 16).

Animal immunizations and Hantaan virus challenge. For immunogenicity studies, groups of 10 CB6F1/J mice were inoculated by tail scarification with $10 \mu\text{l}$ (approximately 10^6 PFU) of each recombinant vaccinia virus. Animals were bled 21 days after immunization, and serum antibody titers to vaccinia virus and Hantaan virus antigen were measured by enzyme-linked immunosorbent assay (ELISA), employing reaction conditions essentially as previously described (20). ELISA antigen consisted of vaccinia virus or Hantaan virus grown in Vero E6 cells and purified by sedimentation in sucrose gradients (17). A second inoculation with the same amount of each virus was administered i.p. 50 days after the first inoculation to half of the mice in each group. After 21 days, all mice were again bled (including the five animals that had received only one inoculation), and serum antibody titers were measured by ELISA.

To assess protection as well as immunogenicity, 8- to 10-week-old outbred female Syrian hamsters (Charles River) were immunized with candidate recombinant vaccine preparations as follows. Primary infections with recombinant vaccinia viruses were accomplished by scarification of the skin in the axillary region with a bifurcated needle and the subsequent addition of $25 \mu\text{l}$ of virus suspensions containing approximately 10^9 PFU per ml. Booster injections of recombinant vaccinia viruses were administered 28 days after the primary immunization by i.p. injection of 0.2 ml of the identical viruses diluted 1:10 in sterile PBS. Recombinant baculovirus-infected SF-9 cells were pelleted by low-speed centrifugation, washed once in PBS, and, after a second centrifugation, resuspended in PBS to a concentration of 10^8 cells per ml. Cells were disrupted by freezing at -70°C and thawing at 37°C , and the resultant suspensions were injected i.m. (0.1 ml into each rear leg). Booster injections were given at 28 days and were identical to the primary injections. Animals were bled by cardiac puncture under anesthesia, and serum antibody responses were measured 1 day before challenge by IFAT (1) and PRNT on Vero E6 monolayers

(16) and 28 days after challenge by IFAT. Hamsters were challenged i.m. approximately 4 weeks after the booster injection (8 weeks post-primary immunization) with 10^3 PFU of Hantaan virus. Hamsters were evaluated as described above.

All experiments with infectious viruses were conducted in the containment facilities at the U.S. Army Medical Research Institute of Infectious Diseases.

RESULTS

Preparation of recombinant viruses. Hantaan virus genes were inserted into the polyhedrin gene of the baculovirus AcNPV or the thymidine kinase gene of two strains of vaccinia virus, the WR mouse neurotropic strain and the Connaught human vaccine strain. Recombinant baculoviruses and vaccinia viruses that contained coding information representing the Hantaan virus S genome segment, the entire M segment, or portions of the M segment encoding only the G1 or the G2 envelope glycoprotein were generated. Because G1 and G2 are encoded in a single, continuous open reading frame and because the exact carboxy terminus of G1 is not known (21), the recombinant expressing only G1 was constructed such that the potential intergenic region between G1 and G2 as well as coding information for the amino-terminal portion of G2 (211 amino acids) were included to allow cleavage at the G1-G2 junction (Fig. 1). For preparation of a recombinant expressing only G2, a restriction fragment containing all of the G2-coding sequences plus 33 amino acids prior to the amino terminus of G2 was inserted into the transfer vectors. The additional nucleotide sequences included an in-frame ATG located 17 codons before the start of G2, which served as a translation initiation codon (Fig. 1).

Expression of Hantaan virus proteins by recombinant baculoviruses. Expression of the complete M segment of Hantaan virus by AcNPV resulted in proteins indistinguishable from authentic Hantaan virus G1 and G2 by immune precipitation and PAGE (Fig. 2). Although insect cells have slightly different glycosylation properties than mammalian cells, any differences in the carbohydrate components of the expressed proteins compared with the authentic proteins (18) were not evident by their electrophoretic migrations.

As previously reported (12), differences in transfer vectors can result in very different levels of foreign gene expression by recombinant baculoviruses. Therefore, we used two different transfer vectors reported to yield high levels of expression, pAcYM1 (13) and pVL941 (10), to construct recombinants expressing the complete M segment of Hantaan virus. Similar levels of G1 and G2 appeared to be produced by the two resultant recombinant viruses, and the recombinant prepared by using the pAcYM1 transfer vector was used in all subsequent experiments.

Expression of gene regions encoding only G1 or G2 similarly resulted in proteins analogous to authentic G1 and G2 (Fig. 2). Although recombinants expressing G1 also had coding information for the first 211 amino acids of G2 and thus could potentially produce a truncated G2 protein of approximately 23,000 daltons, no corresponding polypeptide could be detected by PAGE, suggesting that the partial G2 protein, if it was made at all, was unstable or was not recognized by the antibodies used for immune precipitation. As previously described (22), recombinants containing coding information from the S genome segment of Hantaan virus produced large amounts of N, which could be immune precipitated with Hantaan virus-specific antibodies.

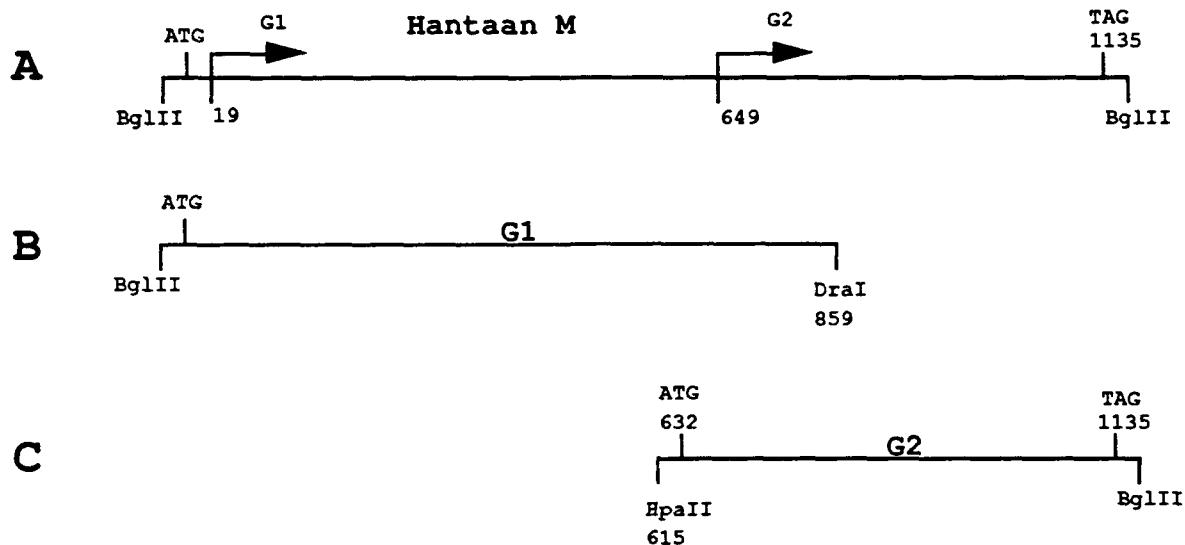


FIG. 1. Hantaan virus M segment genes expressed by baculovirus and vaccinia virus vectors. Restriction fragments containing cDNA representing the entire M segment coding region (A) or the coding region for only G1 (B) or G2 (C) were excised and cloned into the baculovirus and vaccinia virus transfer vectors. Numbers correspond to the amino acids encoded in the M segment with respect to the first ATG initiation codon of the open reading frame.

Expression of Hantaan virus proteins by recombinant vaccinia viruses. Both the Connaught and WR strain recombinant vaccinia viruses expressed the complete Hantaan virus M segment or portions of the M segment encoding only G1 or G2 and yielded proteins indistinguishable from G1 and G2 by PAGE (14). Expression of the S segment of Hantaan virus similarly generated a protein identical to N by PAGE (Fig. 2); however, the level of expression observed was quite low as compared with that of the M segment and was considerably lower than could be achieved in the baculovirus system. With Connaught strain recombinants, immune precipitation of expressed proteins from Vero E6 cell lysates coinfecting with the three vaccinia virus recombinants which individually express G1-, G2-, or N-coding regions resulted in protein profiles closely resembling those obtained by PAGE

of authentic Hantaan virus proteins (Fig. 2). Similar results were obtained by coinfection with recombinants expressing the complete M and S segments of Hantaan virus.

Antigenicity of the expressed proteins. To determine whether the expressed proteins retained antigenic properties similar to those of authentic Hantaan virus proteins, cell cultures infected with baculovirus or vaccinia virus recombinants were screened by IFAT with 20 MAb to the G1 or G2 proteins of Hantaan virus (1). Six MAb to G1, which recognize two distinct antigenic sites, and 14 MAb to G2, which recognize seven distinct sites, were tested against SF-9 cells infected with the baculovirus recombinants or against Vero E6 cells infected with the vaccinia virus recombinants. All of the G1-specific MAb reacted with proteins from recombinants expressing G1 or those expressing both

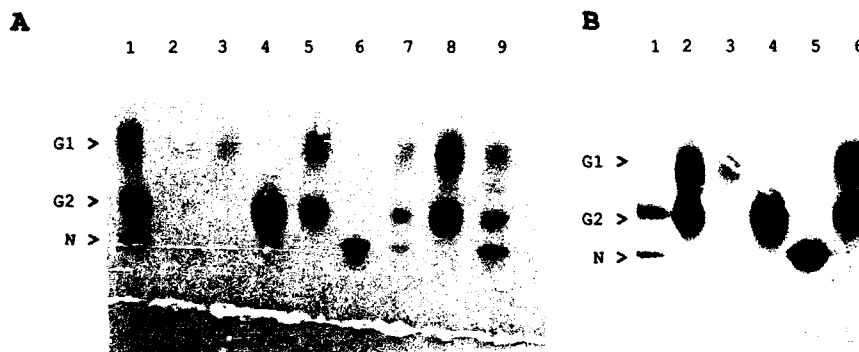


FIG. 2. PAGE of radiolabeled proteins immune precipitated from infected cell lysates. (A) Authentic Hantaan virus proteins were precipitated from Vero E6 cell lysates with anti-Hantaan virus hyperimmune mouse ascitic fluid (lane 1) or normal mouse ascitic fluid (lane 2). Proteins generated by recombinant vaccinia virus expression of the following Hantaan virus genes were precipitated from Vero E6 cell lysates with anti-Hantaan virus hyperimmune mouse ascitic fluid: G1 (lane 3); G2 (lane 4); coinfection with recombinants expressing only G1 and only G2 (lane 5); S (lane 6); coinfection with recombinants expressing only G1, only G2, and S (lane 7); M (lane 8); and coinfection with recombinants expressing M or S (lane 9). (B) Authentic Hantaan virus proteins (lane 1) were precipitated from infected Vero E6 cell lysates with anti-Hantaan virus hyperimmune mouse ascitic fluid. Recombinant baculoviruses expressing the following genes were precipitated from infected *S. frugiperda* cell lysates with anti-Hantaan virus hyperimmune mouse ascitic fluid: M (lane 2), G1 (lane 3), G2 (lane 4), S (lane 5), and M (lane 6). All recombinants were prepared with pAcYM1 transfer vectors except the recombinant expressing M (lane 6) which was prepared with pVL941 transfer vector. The positions of Hantaan virus G1 and G2 and N are indicated with arrowheads.

TABLE 1. Immune responses of mice to vaccinia and Hantaan viruses after one or two immunizations with vaccinia-Hantaan recombinant viruses^a

Immu- nogen	ELISA titer					
	Vaccinia virus antigen			Hantaan virus antigen		
	Primary		Secondary	Primary		Secondary
	Day 21	Day 71	Day 71	Day 21	Day 71	Day 71
CN M	1,600	6,400	>52,100	800	6,400	>12,800
WR M	6,400	12,800	>52,100	800	6,400	>12,800
CN G2	1,600	12,800	>52,100	<100	800	6,400
WR G2	6,400	25,600	>52,100	400	1,600	3,200
CN G1	1,600	6,400	>52,100	100	800	1,600
WR G1	3,200	6,400	>52,100	100	800	3,200
CN S	1,600	6,400	>52,100	<100	<100	200
WR S	6,400	12,800	25,600	<100	<100	<100

^a Animals were immunized with WR or Connaught (CN) strain recombinant vaccinia viruses. Serum samples were collected 21 and 71 days after a primary immunization and 50 days after a secondary immunization (71 days after the primary immunization), and ELISA titers to vaccinia and Hantaan virus antigens were determined. Vaccinia virus ELISA titers are the reciprocals of dilutions with readings of >0.5 at an optical density of 410 nm. Hantaan virus ELISA titers are the reciprocals of dilutions with readings of >0.15 at an optical density of 410 nm after subtraction of values obtained with controls which had the G2 gene inserted backwards with respect to the promoter. Titers were obtained with pooled sera from 10 (primary) or 5 (late primary and secondary) animals, except for the CN M (day 71) group, which had 3 animals, and the WR M (day 71) group, which had 4 animals.

G1 and G2 but not with those expressing only G2 or N or with uninfected cell controls. Similarly, the anti-G2 MAb reacted only with cells infected with recombinants expressing G2 or both G1 and G2. These data indicated that at least the antigenic sites defined by these MAb were conserved on the expressed proteins.

Immunogenicity of the expressed proteins. Sera from mice inoculated once or twice with live recombinant vaccinia viruses were evaluated by ELISA for the presence of antibodies to both vaccinia and to Hantaan virus antigens. Antibody titers of individual animals to vaccinia virus measured at 3 and 10 weeks after the initial immunization indicated that all animals were infected with the recombinant viruses (data not shown). Pooled sera, which reflected results obtained with individual samples, are displayed in Table 1. Antibody responses to Hantaan virus were detected 3 weeks after immunization with sera from animals immunized with all recombinants except Connaught G2, Connaught S, and WR S. The antibody titers observed 10 weeks after immunization were higher in all groups except those animals immunized with the S segment recombinants. Animals that received a second immunization with the recombinant viruses displayed higher antibody titers both to vaccinia virus and to Hantaan virus, again with the exception of the S segment recombinants. Because the animals immunized with the S segment recombinants displayed titers to vaccinia virus that were comparable to all other recombinants, the relatively low S segment expression observed with these viruses is probably responsible for their low-titer responses. In these studies, it was not possible to determine whether there were quantitative differences in the antibody responses elicited by the WR versus the Connaught strain recombinants, because the initial immunizing doses were not the same. However, in separate experiments in which the same amounts of infectious Connaught or WR strain recombinant vaccinia viruses were administered, mice receiving the more virulent WR strain viruses developed slightly

higher and more rapid antibody responses, both to vaccinia virus and to Hantaan virus (data not shown).

To determine the specificities of the antibody responses, sera from mice infected only once as well as sera from mice receiving a second booster infection with each recombinant vaccinia virus were used to immune precipitate radiolabeled Hantaan virus proteins. After one inoculation, mice infected with G1 and G2 recombinants displayed antibodies to both proteins. No qualitative differences in antibody responses could be detected in animals immunized with WR or Connaught strain recombinant viruses. In agreement with results obtained by ELISA, a second immunization with each recombinant virus appeared to boost the antibody responses to Hantaan virus proteins (Fig. 3).

We were unable to evaluate the immunogenicity of the baculovirus-expressed proteins in mice because only low-titer antibody responses (1:40 neutralization titer for mice immunized with the complete M segment recombinant) were elicited, even after two inoculations with recombinant-infected SF-9 cell preparations. We did not determine the reasons for the poor responses in mice; however, in similar experiments with baculovirus-expressed G1 and G2 from another member of the *Bunyaviridae* (Rift Valley fever virus), we were able to induce high-titer antibody responses (20). These data suggest that the lower levels of G1 and G2 produced by the Hantaan virus recombinants, rather than the animal species, may be responsible for the poor antibody response.

Protection of hamsters from infection with Hantaan virus.

An animal model that mimics the symptoms of HFRS has not been reported. Hantaviruses generally cause persistent infections of rodents, with no apparent pathogenicity; however, viral antigens can be detected routinely in selected organs of naturally or experimentally infected animals. To evaluate potential protective immune responses, we developed a Hantaan virus challenge model in hamsters. We found that with a Hantaan virus dose of 1,000 PFU administered i.m., all hamsters were infected and displayed antigen in their lungs (Table 2). Lower virus doses did not infect all animals reproducibly, and higher doses resulted in less detectable antigen in the lungs of animals, possibly reflecting antigen clearance by 28 days postinfection. Therefore, for all protection experiments, animals were challenged with 1,000 PFU of Hantaan virus.

To examine the participation of specific viral epitopes in the protective immune response, MAb recognizing seven different sites on G2 and two on G1 (1) were passively transferred to hamsters which were subsequently challenged with Hantaan virus. Most of the animals receiving neutralizing G2 MAb did not develop antibodies detectable by IFAT after challenge, and only one animal displayed any antigen in its lungs (Table 3). These results suggest that the MAb protected the hamsters from infection. Although the three G1-specific neutralizing MAb recognize the same or closely related antigenic sites (as revealed by competitive inhibition studies; 1), one of these antibodies appeared to completely protect animals from infection, while the other two appeared to offer only limited protection. None of the nonneutralizing antibodies or normal mouse ascitic fluid appeared to prevent animals from developing high-titer antibody responses after challenge, and most animals displayed antigen in their lungs, which together indicate that animals were infected. These data suggest that a neutralizing antibody response alone is sufficient to protect hamsters from infection with Hantaan virus and that neutralizing

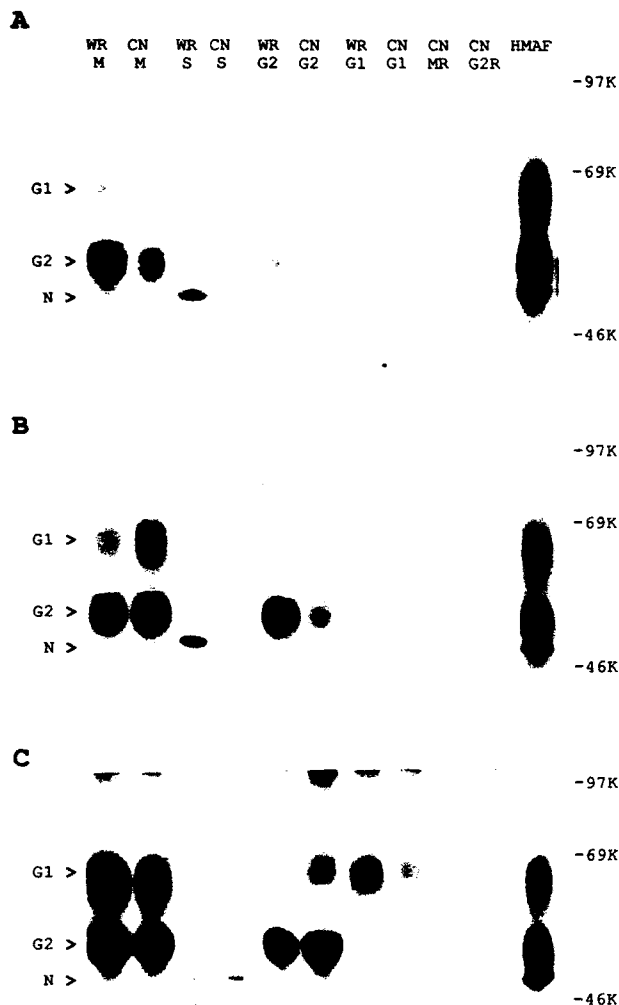


FIG. 3. PAGE of radiolabeled authentic Hantaan virus proteins immune precipitated with sera from mice immunized with WR or Connaught (CN) strain recombinants expressing the complete M (lanes WR M and CN M), S (lanes WR S and CN S), G2 (lanes WR G2 and CN G2), and G1 (lanes WR G1 and CN G1) segments and controls prepared in Connaught strain viruses by inserting M (lane CN MR) or the G2 gene (lane CN G2R) backwards so that the genes could not be expressed. A control immune precipitation with anti-Hantaan virus hyperimmune mouse ascitic fluid (HMAF) was included. Sera were obtained 21 days after a single immunization with each virus (A), 71 days after a single immunization (B), or 50 days after a second booster immunization (71 days after the primary immunization) (C). The positions of Hantaan virus envelope proteins (G1 and G2) and N are indicated with arrowheads. Numbers to the right of autoradiographs refer to ^{14}C molecular weight (thousands) markers which were removed prior to photography.

antibodies to either G1 or G2 can passively confer protection.

To evaluate the ability of the recombinant-expressed proteins to elicit a protective response in hamsters, animals were given two immunizations with each recombinant virus preparation as described in Materials and Methods. Serum antibody titers were measured after each immunization and again after challenge. Animals were killed, and lung sections were examined for the presence of Hantaan virus antigen.

TABLE 2. Hamster challenge model

Virus dose (PFU)	No. infected ($n = 5$) ^a	Antigen in lungs ^b		ELISA titer ^c
		Low	High	
10	3	+++	+++	320
100	4	+	+++	360
1,000	5	++	+++	4,830
10,000	5	+	+++	5,120
100,000	5	0	+	>12,800

^a Infection was determined by detectable antibody response.

^b Lowest (+) and highest (+++) amount of antigen detected in lung sections of individual hamsters.

^c Average titer for all infected animals in each group.

Animals receiving the baculovirus or vaccinia virus recombinants expressing the complete M segment developed neutralizing antibody titers before challenge and did not display antigen in their lungs after challenge (Table 4). Most animals immunized with the G1, G2, or M segment recombinants did not display antibodies detectable by indirect immunofluorescence prior to challenge; therefore, the presence of IFAT-detectable antibodies after challenge suggested that the animals became infected with the Hantaan virus challenge. No postchallenge, Hantaan-specific antibodies were observed in the sera of animals immunized with the complete M segment recombinant preparations, suggesting that they were protected from infection. Similar results were obtained with hamsters immunized with authentic Hantaan virus preparations that were inactivated by irradiation. In contrast, animals immunized with preparations of cells infected with baculovirus recombinants expressing only G1- or G2-coding regions did not develop neutralizing antibodies before challenge, and some of the animals became infected after challenge, as indicated by IFAT-detectable serum antibodies. However, none of the animals displayed detectable

TABLE 3. Passive protection of hamsters with MAbs to Hantaan virus G1 and G2 proteins

Antigenic site (MAb) ^a	No. positive/total ^b		Antigen in lungs
	Antibody		
	IFAT	PRNT	
Neutralizing			
G2a (HCO2)	1/4	0/4	0/4
(16E6)	1/4	1/4	1/4
G2c (11E10)	1/4	1/4	0/4
G1b (3D5)	0/4	0/4	0/4
(2D5)	4/4	4/4	1/4
(16D2)	4/4	4/4	2/4
Nonneutralizing			
G2b (EBO6)	4/4	4/4	4/4
G2d (3D7)	4/4	4/4	3/4
G2e (20D3)	4/4	4/4	3/4
G2f (23G10)	3/3	3/3	3/3
(GDO5)	4/4	4/4	3/4
(8E10)	4/4	4/4	3/4
G2g (JDO4)	4/4	4/4	4/4
G1a (6D4)	3/3	3/3	3/3
(8B6)	3/3	3/3	1/3
Normal ascitic fluid	5/5	5/5	5/5

^a Antigenic sites G1a and G1b and G2a to G2f and the characterization of individual MAb were described by Arikawa et al. (1).

^b Titers $\geq 1:64$ at 22 days postchallenge.

TABLE 4. Challenge of hamsters immunized with authentic or expressed Hantaan virus proteins

Immunogen	No. positive/total ^a					
	Prechallenge antibody				Postchallenge	
	PRNT		IFAT		Antigen in lungs	Antibody (IFAT)
	1°	2°	1°	2°		
Baculovirus						
M	4/5	5/5	2/5	2/5	0/5	0/5
G1	0/5	0/5	0/5	0/5	0/5	2/5
G2	0/5	0/5	0/5	0/5	0/5	4/5
S	0/5	0/5	5/5	5/5	0/5	5/5
Uninfected cell control	0/5	0/5	0/5	0/5	4/5	4/5
Vaccinia virus						
WR-M	5/5	5/5	0/5	0/5	0/5	0/5
Con-M	2/5	5/5	0/5	0/5	0/4	0/4
Con-G1	0/5	0/5	0/5	0/5	1/5	5/5
Con-G2	0/5	0/5	0/5	0/5	2/5	2/5
Con-S	0/5	0/5	0/5	0/5	3/5	5/5
Hantaan virus ^b						
Virion	0/5	5/5	4/5	4/5	0/5	0/5
Purified virion	0/10	7/10	7/10	7/10	0/9	0/9
Infected cells	0/5	2/5	0/5	0/5	1/5	1/5
Uninoculated control	0/5	0/5	0/5	0/5	5/5	5/5

^a Number positive/number inoculated with PRNT titers $\geq 1:10$ or IFAT titers $\geq 1:16$ after primary (1°) or secondary (2°) immunization.

^b Inactivated by irradiation with ⁶⁰Co.

antigen in its lungs. Similar results were obtained with the vaccinia virus recombinants producing only G1 or only G2; i.e., hamsters did not develop neutralizing antibody responses and some of the animals became infected and displayed Hantaan virus antigens in their lungs.

Although antibodies to Hantaan virus N are not known or presumed to be involved in neutralization, animals immunized with a preparation of cells infected with the baculovirus recombinant expressing the S segment did not have antigen in their lungs after challenge. The same degree of apparent protection was not observed with vaccinia virus recombinants expressing S. Although no attempt was made to quantitate the exact amount of antigen each animal received (and in fact it would be impossible to do so with the replicating recombinant vaccinia viruses), it is likely that the large amount of expressed N produced by the recombinant baculovirus (22), as compared with that produced by the recombinant vaccinia virus, provided a greater immunizing antigen dose. Any suggestion that the recombinants induce protective immune mechanisms other than neutralization (e.g., a cell-mediated response) must await further investigation.

In order to confirm our initial observations that the absence of antigen in the lungs of hamsters and our inability to detect antibodies by IFAT after challenge indicated protection, additional groups of hamsters (five per group) were immunized either once or twice with the baculovirus recombinants producing G1 and G2, G1 only, G2 only or N, or with the Connaught and WR strain vaccinia virus recombinants expressing both G1 and G2. Selected sera from each group were used for immune precipitation of authentic Hantaan virus proteins. The sera selected for study represented those within each group which were protected from challenge and

those which remained susceptible to infection on the basis of our established criteria for estimating infection, i.e., the best and worst protective responses from each group. Animals that did not become infected should possess antibodies only to the expressed protein(s) with which they were immunized both before and after challenge, but animals that did become infected should exhibit demonstrable antibodies both to the protein(s) with which they were immunized before challenge as well as all three Hantaan virus structural proteins after challenge. In these experiments, all animals immunized with preparations of recombinant baculovirus-infected cells expressing both G1 and G2 genes appeared to be protected from infection; i.e., no antigen was apparent in lungs and there was no IFAT-detectable, Hantaan virus-specific antibody after challenge. Sera from hamsters receiving either one or two immunizations with these recombinant preparations had antibodies to G1 and G2 both before and after challenge, but not to N, confirming that the animals were not infected with Hantaan virus (Fig. 4A, lanes +M-1° and +M-2°). The predominant response appeared to be to G2. In contrast, most animals immunized once with preparations containing only baculovirus-expressed G2 did not appear to be protected, although the absence of antigen in the lungs of some animals suggested partial protection. Serum from an animal displaying no antigen in its lungs and antibodies only to G2 before challenge still exhibited immune precipitation of G1, G2, and N after challenge, indicating that the animal was infected with Hantaan virus (Fig. 4A, lane +G2-1°). After two immunizations with the same preparation followed by challenge, the serum from an animal with no IFAT-detectable antibody and no antigen in its lungs was found to immune precipitate only G2, thus the animal was protected (Fig. 4A, lane +G2-2°). Two immunizations with the G2 recombinant vaccinia virus did not protect all animals from infection, however, as indicated by immune precipitation of all three Hantaan virus proteins by antibodies contained in the serum of an animal which did display antigen in its lungs despite two inoculations (Fig. 4A, lane -G2-2°). Therefore, immunization with the individual G2 protein can protect some animals from infection but not as effectively as does immunization with expressed G1 and G2 together. In this study, the baculovirus-expressed G1 protein did not protect any animals from Hantaan virus infection, even after two immunizations (Fig. 4B).

As observed in our previous experiments, two immunizations with preparations containing baculovirus-expressed N protected five out of five animals in that no Hantaan virus antigen could be detected in lungs and only N-specific antibodies were observed both before and after challenge, confirming that the N protein induced some sort of protective response in hamsters (Fig. 4B, lane +S-2°). Also in agreement with our previous experiments, most animals immunized with either the Connaught or WR strain vaccinia virus recombinants expressing both G1 and G2 appeared to be protected. One animal immunized only once with a WR strain recombinant developed IFAT-detectable antibody to Hantaan virus after challenge but did not exhibit detectable antigen in its lungs. This animal had antibodies to G1, G2, and N after challenge, indicating that it was infected (Fig. 4C, lane -WR M-1°). All other sera examined contained antibodies only to the envelope glycoproteins both before and after challenge, supporting the conclusion that the recombinant proteins are able to induce an immune response in hamsters, which protects them from subsequent infection with Hantaan virus.

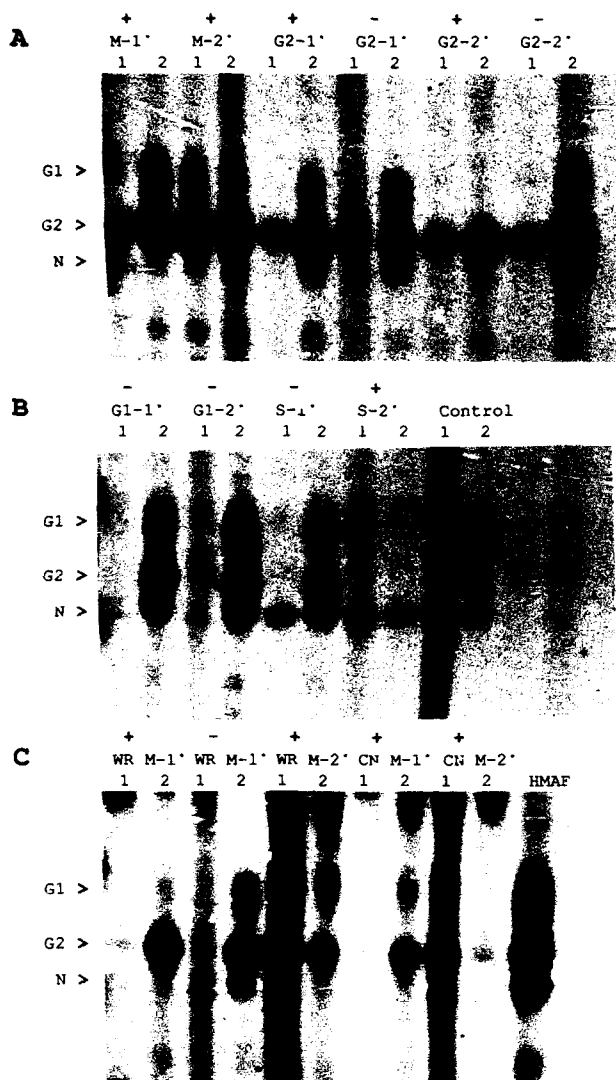


FIG. 4. PAGE of radiolabeled authentic Hantaan virus proteins immune precipitated with sera from hamsters immunized one time (1°) or two times (2°) with baculovirus or vaccinia virus recombinants and challenged with Hantaan virus. (A) Sera obtained from animals immunized with cells infected with baculovirus recombinants expressing M or G2. (B) Sera obtained from animals immunized with cells infected with baculovirus recombinants expressing G1 or S or from control animals immunized with uninfected *S. frugiperda* cells. (C) Sera obtained from animals immunized with WR strain recombinants or Connaught (CN) strain recombinants expressing M. A control immune precipitation with anti-Hantaan virus hyperimmune mouse ascitic fluid (HMAF) was included. Lanes 1. Proteins precipitated with hamster sera obtained before challenge; lanes 2. proteins precipitated with hamster sera obtained after challenge. +, Animals that appeared to be protected from infection, as determined by the absence of serum antibody titers and detectable Hantaan virus antigen in their lungs after challenge; -, animals that did not appear to be protected.

DISCUSSION

Expression of the genes of pathogenic viruses by eucaryotic virus vectors, such as vaccinia virus and AcNPV, has the potential for providing a safe and efficient means of generating immunogens for vaccine development. We were able to express the genes encoding the G1 and G2 envelope

glycoproteins and the N protein of Hantaan virus in both systems. The expressed proteins were found to closely resemble authentic Hantaan virus proteins and were antigenically identical to those proteins within the limits of our panel of MAb. Moreover, the expressed proteins were able to induce antibodies in test animals which could both recognize authentic viral proteins in serological assays and neutralize viral infectivity.

The absence of HFRS-like disease in animals made it difficult to evaluate the ability of our potential vaccines to moderate disease; however, we were able to use a much more stringent test of experimental vaccine efficacy of the recombinant-expressed proteins, i.e., protection from Hantaan virus infection. As demonstrated by passive protection studies, a humoral response to either of the envelope glycoproteins was sufficient to protect against challenge, suggesting that immunization with expressed G1 or G2 proteins might elicit a protective immune response. However, we found that immunization with recombinants expressing the entire M segment, i.e., both G1 and G2, was much more effective at eliciting anti-Hantaan virus antibodies and protecting animals from Hantaan virus infection than immunization with those expressing either G1 or G2 genes separately. Whether this is due to an additive effect of antibodies to both proteins or to some other reason, such as an interaction of the two proteins which results in the formation or stabilization of conformationally dependent antigenic sites, was not resolved.

Previous reports indicated that a cell-mediated response to Hantaan virus may also be involved in protection, but the Hantaan virus component(s) eliciting the response was not identified (2, 3). In our studies, the ability of expressed N to protect animals from Hantaan virus challenge suggests that this protein can elicit a nonneutralizing, perhaps cell-mediated, protective immune response. Although there are previous examples of cell-mediated responses to internal viral antigens protecting animals from lethal challenge (8), our results were somewhat surprising in that virus infection was apparently prevented, as determined by the absence of detectable antibodies to any viral protein other than N following challenge. Additional information is required to define the important aspects of immunity to Hantaan virus; however, our data suggest that whatever the mechanism(s), our recombinant-expressed proteins are clearly capable of inducing a response that can protect animals from infection with Hantaan virus.

One goal of studies such as ours is the development of a vaccine to prevent HFRS. Although both the baculovirus and vaccinia virus expression systems yielded products effective in inducing immune responses in animals, each has advantages and disadvantages for use in the development of a vaccine suitable for humans. Recombinant vaccinia viruses offer the advantages of a live virus vaccine without the danger of reversion to the virulent form of the virus. The major disadvantage of a recombinant vaccinia virus vaccine is that it is an infectious agent and, therefore, could cause vaccine-related complications (albeit rarely), such as those observed during its use as a smallpox vaccine. Unlike vaccinia virus and Hantaan virus, baculoviruses cannot infect mammalian cells, so a vaccine developed from recombinant AcNPV offers the advantages of a traditional inactivated vaccine, without the potential risk of incomplete inactivation. Furthermore, production of such a vaccine could be accomplished safely under minimal containment conditions. Several disadvantages to a baculovirus-derived vaccine also stem from its ability to replicate only in insect

cells, for example, the need to recover and purify the expressed proteins from infected cell cultures prior to their use in humans.

The data presented here demonstrate the feasibility of using expressed Hantaan virus proteins to immunize animals to Hantaan virus. These studies should provide a basis for future exploitation of recombinant-expressed Hantaan virus proteins as potential human vaccines.

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